REMARKS

This Amendment and Response (and accompanying Request for an Extension of Time to Respond) are in reply to the Office Action mailed May 21, 2003 in the subject application. Claims 1 and 3-9 stand rejected. Upon entry of the proposed amendments, claims 9-13 will be pending, claims 10-13 being newly added.

Entry of the above made amendments and reconsideration of the subject application in view thereof as well as the following Remarks is respectfully requested.

REJECTIONS UNDER SECTION 112

Claims 1 and 3-8 were rejected on the basis that the specification does not enable all "vitamin D2 derivatives" and "vitamin D3 derivatives." Claims 1, 3-6 and 8 also stand rejected on the basis that the phrases "vitamin D2 derivative" and "vitamin D3 derivative as recited in claim 1 (from which the remaining pending claims depend) render claim 1 indefinite.

Applicants appreciate the examiner's acknowledgement that the specification enables the vitamin D compounds disclosed in the specification at page 3, line 11 through page 4, line 5.

As indicated above and as mentioned during Applicants' telephone interview with the examiner on July 29, 2003, Applicants have amended dependent claim 9 to be in independent form. Claim 9, originally depending from claim 1 (through claim 8), recited a specific vitamin D compound within the scope of Applicants' invention and disclosed in the subject application as originally filed. Specifically, vitamin D 1α, 25-dihydroxy-19-nor ergocalciferol is taught at least on page 2, line 24 of the specification as originally filed. New claims 10, 11 and 12 paralleling original claims 3, 4 and 5, respectively depend from claim 9. Support for new claim 13 appears in the specification as filed at least at page 1, line 31. No new matter has been added by these amendments.

In addition, as indicated above, Applicants withdraw claims 1 and 3-8 from further consideration in the subject application and do so without prejudice to the filing of a new application consistent with 35 U.S.C.

In that claim 9 was not rejected as indefinite, the subject application, including new claims 10-13, meets the enablement and definiteness (and all of the other) requirement of Section 112 and therefore the outstanding rejections should be withdrawn.

REJECTION UNDER SECTION 103

Claims 1-9 were rejected as obvious over Knutson, et al (the '473 patent) and the ZEMPLAR monograph from the Physicians' Desk Reference April 1998, pages 478-480).

Applicants again disagree with the rejection of the claims on the teachings of these two references.

Claims 1-8 have been cancelled without prejudice as indicated above.

Claim 9 as amended recites a method for treating ICU-associated hypocalcemia comprising the step of "administering a therapeutically effective amount of 1α, 25-dihydroxy-19-nor ergocalciferol."

In Applicants' previous response, a table was provided to summarize several distinct disease states. Chapter 357, pages 2165-2169 of Harrison's Principles of Internal Medicine (13th ed.), cited by the examiner, concurs with the summary of information provided by the table.

In addition, reference may be made to the authoritative (and voluminous) reference treatis entitled ***Vitamin D*** (Feldman, Glorieux, and Pike (Eds.), Academic Press, 1997, pages 3-11. A copy of the first chapter of ***Vitamin D*** (entitled HISTORICAL OVERVIEW) is enclosed for the examiner's convenience. Applicants also provide below a revision of the table to clarify the distinctions between Applicants' invention as presently claimed and the disclosures of the cited references.

As will be appreciated by a side-to-side comparison of the earlier table, the revised table below replaces the arrows with words but still represents certain biochemical manifestations associated with these disease states.

Disease condition	Serum Ca	Serum P	Serum PTH	Serum Vitamin D
ICU-associated Hypocalcemia	Depressed	Depressed/ Elevated/Normal	**Normal**	**Normal**
Secondary hyperparathyroidism	Depressed	Elevated	**Elevated**	**Depressed**
Vitamin D deficiency	Depressed	Depressed	Elevated	Depressed
Pseudo-hypoparathyroidism	Depressed	Elevated/Normal	Elevated	Depressed

Further to Applicants' previously submitted remarks regarding the conditions described in the earlier table, Applicants further submit the following.

The outstanding Office Action states on page 6:

> "It would have been obvious for one of ordinary skill in the art at the time the invention was made to employ 1,25-dihydroxy Vitamin D3 or 1,25-dihydroxy-19-nor ergocalciferol (sic) daily in a method to treat ICU-associated hypocalcemia for 1-4 weeks."

> "One of ordinary skill in the art would have been motivated to employ 1,25 di-hydroxy vitamin D3 or 1,25 dihydroxy-19-nor ergocalciferol (sic) daily in a method to treat ICU associated hypocalcemia for 1-4 weeks because 1,25 –dihydroxy Vitamin D3 or 1,25 dihydroxy-19-nor ergocalciferol (sic) are known in the art to be useful in a method to treat the symptoms of ICU-associated hypocalcemia (i.e., ***hypocalcemia and an increased level of PTH)***." (Emphasis added)

This statement clearly reflects a misunderstanding of the basic manifestations of ICU-associated hypocalcemia. As stated in the revised table above and taught in Harrison at page 2165, second paragraph, right side, serum PTH may be **normal** in***ICU-associated*** hypocalcemia.

Moreover, the subject application further demonstrates the unique context of ICU-associated hypocalcemia at page 1, lines 24-34 which state:

> Currently, hypocalcemia in an intensive care unit (ICU) setting is either not treated or it is treated only when the medical professional judges it to be of life threatening severity. Existing treatment modalities for hypocalcemia in this setting are limited to intravenous (IV) infusions of inorganic (e.g., CaCl2) or organic (e.g., calcium gluconate) salts. The problems associated with the administration of calcium salts are (a) IV calcium infusions have attendant risk of cardiotoxicity, (b) IV calcium infusions only treat the manifestations of the abnormality, i.e., low ionized calcium, not the metabolic cause of the abnormality, (c)

because ICU-related hypocalcemia reflects a blood/tissue maldistribution of calcium and not a net calcium loss, the administration of calcium may cause total calcium overload, and (d) calcium infusions are given as a "sliding scale" (increasing degrees of hypocalcemia relative to increasing doses of IV calcium)."

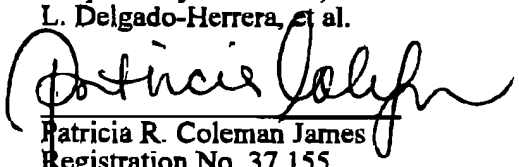
In conjunction with the table, the deficiencies of treatments that may be suggested by the cited prior art become plain.

CONCLUSION

Reconsideration of the subject application and allowance thereof are respectfully requested.

The examiner is encouraged to telephone the undersigned to facilitate resolution of any outstanding issues and allowance of the subject application.

Respectfully submitted,
L. Delgado-Herrera, et al.



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CHAPTER 1

Historical Overview

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I. DISCOVERY OF THE VITAMINS

A. Early Nutritional Views

The field of nutrition was largely dominated in the nineteenth century by German chemists, led by Justus von Liebig [1]. They taught that adequacy of the diet could be described by an analysis of protein, carbohydrate, fat, and mineral. Thus, a diet containing 12% protein, 5% mineral, 10–30% fat, and the remainder as carbohydrate would be expected to support normal growth and reproduction. This view remained largely unchallenged until the very end of the nineteenth century and the beginning of the twentieth century [2–5]. However, evidence opposing this view began to appear. One of the first was the famous study of Eijkman who studied prisoners in the Dutch East Indies maintained on a diet of polished rice [6]. A high incidence of the neurological disorder beri-beri was recorded in these inmates. Eijkman found that either feeding whole rice or returning the hulls of the polished rice could eliminate beri-beri. Eijkman reasoned that polished rice contained a toxin that was somehow neutralized by the rice hulls. Later, a colleague, Grijns [7], revisited the question and correctly demonstrated that hulls contained an important and required nutrient that prevented beri-beri.

Other reports revealed that microorganic nutrients might be present. The development of scurvy in mariners was a common problem. This disease was prevented by the consumption of limes on British ships (hence, the term "Limey" to describe British sailors) and sauerkraut and fruits on other ships. This led Holst and Fröhlich to conclude that scurvy could be prevented by a nutrient present in these foods [8]. Experiments by Lunin, Magendie, Hopkins, and Funk showed that a diet of purified carbohydrate, protein, fat, and salt is unable to support growth and life of experimental animals [2–5]. This suggested that some unknown or vital factor present in natural foods was missing from the purified diets. Hopkins developed a growth test in which natural foods were found to support rapid growth of experimental animals whereas purified materials could not [3]. Funk had found similar results for the prevention of neuritis and reasoned that there were "vital amines" present in foods from natural sources and actually provided the basis for the term "vitamins" used later to describe essential micronutrients [5].

B. McCollum and Osborne and Mendel's Discovery of Vitamin A and B Complex

A key experiment demonstrating essential micronutrients was one carried out at the Wisconsin Agricultural

Experiment Station, engineer d by Stephen Moulton Babcock and carried out by E. B. Hart supported by McCollum and Steenbock [9]. Herds of dairy cows were maintained on a diet composed individually only of corn, oats, or wheat or were fed a mixture of all of these grains, all receiving the same amount of carbohydrate, protein, fat, and salts and all providing equal analysis according to the German chemists [1]. The animals on the corn diet did very well, produced milk in large amounts, and reproduced normally. Those on the wheat diet failed to thrive and soon were unable to reproduce or lactate. The oat group was found to be intermediate between the corn and wheat groups, and the mixture approximated the growth and reproduction found with corn. Yet all these diets had the same proximate analysis.

The conclusion of the Wisconsin Experiment Station study was that there are unknown nutrients present in corn and not found in wheat that are essential for life and reproduction. This led E. B. Hart, Chairman of Biochemistry at Wisconsin, to conceive that a search for these nutrients must begin. Professor McCollum was asked to search for these nutrients using small experimental animals. McCollum and Davis demonstrated there was present in butter fat a substance that prevented xerophthalmia and was also required for growth. They termed this "a lipin-soluble growth factor" [10]. McCollum later named this factor "vitamin A" [11]. This substance was absent from lard and other fats but was found in large amounts in cod liver oil. In constructing the diets, McCollum obtained the carbohydrates and salts from milk whey which, unknown to him, supplied the vitamin B complex group of micronutrients that permitted him to observe a vitamin A deficiency. McCollum at Wisconsin [11] and Osborne and Mendel [12] at the Connecticut Experiment Station carried out experiments in which cod liver oil was used as a source of fat in the diet but the minerals were supplied from pure chemicals mixed to approximate the mineral composition of milk. Starch or sugar was used as the carbohydrate. These animals developed a different group of symptoms, namely, neuritis, which could be cured by the provision of the milk components. McCollum and Osborne and Mendel correctly concluded that this activity was due to a different micronutrient called "vitamin B." This ushered in the concept of the organic micronutrients known as vitamins.

C. History of Rickets

The disease rickets was very likely known in antiquity but was described in the fifteenth century as revealed by later writings. Whistler first provided a clear description of rickets in which the skeleton was poorly mineralized and d formed [13]. Rick ts undoubtedly in ancient times appeared only on rare occasions and hence was not considered a problem. How ver, at th end of the nineteenth century, the industrial revolution had taken place: a highly agrarian population had becom urbanized, and smoke from the industrial plants polluted the atmosphere. Thus, in low sunlight countries such as England, rickets appeared in epidemic proportions. In fact, it was known as the English Disease [14]. Some r ports of the beneficial action of cod liver oil had appeared. However, they were not given scientific credence.

With the discovery of the vitamins, Sir Edward Mellanby in Great Britain began to reason that rickets might also be a disease caused by a dietary deficiency [15]. Mellanby fed dogs a diet composed primarily of oatmeal, which was the diet consumed where the incid nce of rickets was the highest (i.e., Scotland). McCollum inadvertently maintained the dogs on oatmeal indoors and away from ultraviolet light. The dogs developed severe rickets. Learning from the experiments of McCollum, Mellanby provided cod liver oil to cure or prevent the disease. Mellanby could not decide whether the healing of rickets was due to vitamin A known to be present in the cod liver oil or whether it was a new and unknown substance. Therefore, the activity of healing rickets was first attributed to vitamin A.

D. Discovery of Vitamin D

McCollum, who had moved to Johns Hopkins from Wisconsin, continued his experiments on the fat-soluble materials. McCollum used aeration and heating of cod liver oil to destroy the vitamin A activity or the ability to support growth and to prevent xerophthalmia [16]. However, cod liver oil treated in this manner still retained the ability to cure rickets. McCollum correctly reasoned that the activity in healing rickets was due to a new and heretofore unknown vitamin which he termed "vitamin D." On the basis of the experiments of McCollum and of Mellanby, vitamin D became known as an essential nutrient.

II. DISCOVERY THAT VITAMIN D IS NOT A VITAMIN

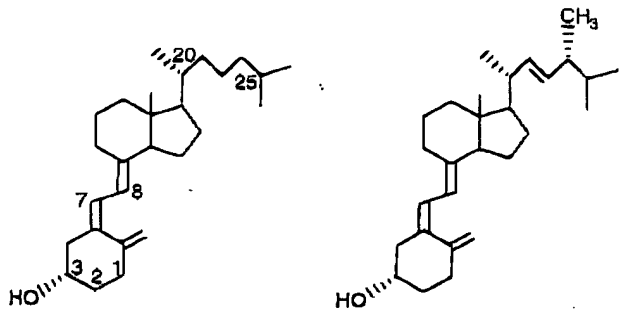
At the same time that Sir Edward Mellanby was carrying out the experiments in dogs, Huldshinsky [17] and Chick *et al.* [18] independently found that rickets in children could be pr vented or cured by exposing them to sunlight or to artificially induced ultraviolet

light. Thus, the curious findings were that sunlight and ultraviolet light somehow equaled cod liver oil. These strange and divergent results required resolution.

Steenbock and Hart had noted the importance of sunlight in restoring positive calcium balance in goats [19]. At Wisconsin, with McCollum carrying out experiments in small experimental animals (i.e., rats), Steenbock was required to work with larger animals. Steenbock then began to study goats because they would consume less materials and could serve as better experimental animals than cows. Steenbock began to study the calcium balance of lactating goats and found that those goats maintained outdoors in the sunlight were found to be in positive calcium balance, whereas those maintained indoors lost a great deal of their skeletal calcium to lactation [19]. Steenbock and Hart, therefore, noted the importance of sunlight on calcium balance. This work then undoubtedly led Steenbock to realize that the ultraviolet healing properties described by Huldschinky might be related to the calcium balance experiments in goats. By irradiating the animals and diets, Steenbock and Black found that vitamin D activity could be induced and rickets could be cured [20]. A similar finding was reported soon thereafter by Hess and Weinstock [21]. Steenbock then traced this to the nonsaponifiable fraction of the lipids in foods [22]. He found that ultraviolet light activated an inactive substance to become a vitamin D active material. Thus, ultraviolet light could be used to irradiate foods, induce vitamin D activity, and fortify foods to eliminate rickets as a major medical problem. This discovery also made available a source of vitamin D for isolation and identification.

III. ISOLATION AND IDENTIFICATION OF NUTRITIONAL FORMS OF VITAMIN D

From irradiation of mixtures of plant sterols, Windaus and colleagues isolated a material that was active in healing rickets [23]. This substance was called "vitamin D_1," but its structure was not determined. Vitamin D_1 proved to be an adduct of tachysterol and vitamin D_2, and thus vitamin D_1 was actually an error in identification. The British group led by Askew was successful in isolating and determining the structure of the first vitamin D, vitamin D_2 or ergocalciferol, from irradiation of plant sterols [24]. A similar identification by the Windaus group confirmed the structur of vitamin D_2 [25]. Windaus and Bock also isolated the precursor of vitamin D_3 from skin, namely, 7-dehydrocholesterol [26]. Furthermore, 7-dehydrocholesterol was synthesized [27]



Cholecalciferol Ergocalciferol

FIGURE 1 Nutritional forms of vitamin D.

and converted to vitamin D_3 (cholecalciferol) as identified by the Windaus group [28]. Thus, the structures of nutritional forms of vitamin D became known (Fig. 1). Windaus and Bock, having isolated 7-dehydrocholesterol from skin, provided the presumptive evidence that vitamin D_3 is the form of vitamin D produced in skin, a discovery that was later confirmed by the chemical identification of vitamin D_3 in skin by Esvelt *et al.* [29] and of a previtamin D_3 in skin by Holick *et al.* [30]. Synthetic vitamin D as produced by the irradiation process replaced the irradiation of foods as a means of fortifying foods with vitamin D and was also rapidly applied to a variety of diseases including rickets and tetany and in the provision to domestic animals such as chickens, cows, and pigs.

Windaus' group provided chemical syntheses of the vitamin D compounds, confirming their structures and thus ending the era of the isolation and identification of nutritional forms of vitamin D and making them available for the treatment of disease. For his contributions, Windaus received the 1928 Nobel Prize in chemistry.

IV. DISCOVERY OF THE PHYSIOLOGICAL FUNCTIONS OF VITAMIN D

A. Intestinal Calcium and Phosphorus Absorption

Besides bone mineralization, the earliest discovered function of vitamin D is its important role in the absorption and utilization of calcium. The first report of this finding was in the early 1920s by Orr and colleagues [31]. Kletzien *et al.* [32] demonstrated that vitamin D plays an important role in the utilization of calcium from the diet, and a number of experiments were carried out

on the utilization of calcium and phosphorus from cereal diets. Nicolaysen was responsible, however, for demonstrating unequivocally the role of vitamin D in the absorption of calcium and independently of phosphorus from the diet [33]. Nicolaysen also followed the early work of Kletzien *et al.* [32] in which animals adapted to a low calcium diet were better able to utilize calcium than were animals on an adequate calcium diet. This work was confirmed by Nicolaysen, who postulated the existence of an "endogenous factor" that would inform the intestine of the skeletal needs for calcium [34]. This endogenous factor later proved to be largely the active form of vitamin D, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] [35].

B. Mobilization of Calcium from Bone

For many years, investigators have attempted to show that vitamin D plays a role directly on the mineralization process of the skeleton. However, early work by Howland and Kramer [36], later work by Lamm and Neuman [37], and more recent work by Underwood and DeLuca [38] demonstrated very clearly that vitamin D does not play a significant role in the actual mineralization process of the skeleton but that the failure to mineralize the skeleton in vitamin D deficiency is due to inadequate levels of calcium and phosphorus in the plasma. Thus, the action of vitamin D in mineralizing the skeleton and in preventing hypocalcemic tetany is the elevation of plasma calcium and phosphorus [39]. These discoveries laid to rest the concept of a role of vitamin D in mineralization. However, Carlsson [40] and Bauer *et al.* [41] were the first to realize that a major function of vitamin D is to induce the mobilization of calcium from bone when required. Thus, in animals on a low calcium diet, the rise in serum calcium induced by vitamin D is the result of actual mobilization of calcium from bone [42]. This important function is known to be essential for the provision of calcium to meet soft tissue needs, especially those of nerves and muscle, on a minute-to-minute basis when it is in insufficient supply from the diet. It is unknown whether the ability of vitamin D to mobilize calcium from bone is osteoclastic-mediated or whether it is due to a transport of calcium across osteoblastic and bone lining cell membranes into the plasma compartment [43]. It is clear, however, that both vitamin D and parathyroid hormone are required for this function [44]. Furthermore, it is clear that vitamin D plays an important role in osteoclastic-mediated bone resorption [45], which is c rtainly the first event in bone remodeling and an essential event in bone mod ling [46]. The actions of vitamin D and parathyroid hormone on osteoclastic-mediated bone resorption are independ nt of one another [47].

C. Renal Reabsorption of Calcium and Phosphorus

A final site of vitamin D action to elevate plasma calcium is in the distal renal tubule. Although experiments were suggestive of a role for vitamin D in increasing renal tubule reabsorption of calcium, a clear demonstration of this did not occur until the late 1980s at the hands of Yamamoto *et al.* [48]. The renal tubule reabsorbs 99% of the filtered calcium even in the absence of vitamin D. However, reabsorption of the last 1% of the filtered load requires both vitamin D and parathyroid hormone. Thus, these agents work in concert in the renal reabsorption of calcium as well as in the mobilization of calcium from bone. Both agents are required to carry out this function.

D. Discovery of New Functions of Vitamin D

With discovery of the receptor for the vitamin D hormone (described in Section V,G below) cam the surprising result that this receptor could be found in a variety of tissues not previously appreciated as targets of vitamin D action. It localizes in the distal renal tubule cells, enterocytes of the small intestine, bone lining cells, and osteoblasts in keeping with its known role in calcium metabolism [49,50]. However, its appearance in tissues such as parathyroid gland, islet cells of the pancreas, cells in bone marrow (i.e., promyelocytes), lymphocytes, and certain neural cells raised the question of whether the functions of vitamin D might be broader than previously anticipated [49,50]. As a result of those findings, new functions of vitamin D have been found. For example, vitamin D plays a role in causing differentiation of promyelocytes to monocytes and the subsequent coalescing of the monocytes into multinuclear osteoclast precursors and ultimately into active osteoclasts [51,52]. Suppression of parathyroid cell growth and suppression of parathyroid hormone gene expression represent other new vitamin D actions [53,54]. In keratinocytes of skin, vitamin D appears to play a role in suppression of growth and in cellular differentiation [55]. Likely, discoveries of many new functions of $1,25(OH)_2D_3$ will be made and are well on their way, as described in later chapters of this volume.

V. DISCOVERY OF THE HORMONAL FORM OF VITAMIN D

A. Early Work of Kodicek

The true pioneer of vitamin D metabolism was Egan Kodicek working at the Dunn Nutritional Laboratory in Cambridge. Kodicek used a bioassay at first to study the fate of the vitamin D molecule and found that much vitamin D was converted to biologically inactive products [56]. Clearly, however, this approach of assaying vitamin D activity following administration of known doses of vitamin D was of limited value in determining metabolism.

B. Radiolabeled Vitamin D Experiments

Professor Kodicek then began to synthesize radiolabeled vitamin D_2. Unfortunately, the degree of labeling was not sufficient to permit the administration of truly physiological doses of vitamin D. Nevertheless, Professor Kodicek continued investigations into this important area. At the conclusion of 10 years of work, he concluded that vitamin D was active without metabolic modification and that the metabolites that were found were biologically inactive [57]. This conclusion was reached even as late as 1967, when it was concluded that vitamin D_3 itself was the active form of vitamin D in the intestine [58]. However, chemical synthesis of vitamin D_3 of high specific activity in the laboratory of the author proved to be of key importance in the demonstration of biologically active metabolites [59]. By providing a truly physiological dose of vitamin D, it could be learned that the vitamin D itself disappeared and instead polar metabolites could be found in the target tissues [60]. The polar metabolites proved to be more biologically active and acted more rapidly than vitamin D itself [61]. Thus, presumptive evidence of conversion of vitamin D to active forms had been obtained as early as 1967.

C. Isolation and Identification of the Active Form of Vitamin D

By 1968, the first active metabolite of vitamin D was isolated in pure form and chemically identified as 25-hydroxyvitamin D_3 ($25OHD_3$) [62]. Its structure was confirmed by chemical synthesis [63] that provided it for study to the medical and scientific world. For a couple of years, 25OHD was visualized as the active form of vitamin D. However, when it was synthesized in radiolabeled form, it was found to be rapidly metabolized to yet more polar metabolites [64]. By this time, the Kodicek laboratory reawakened their interest in metabolism of vitamin D and began to study the metabolism of 1α-tritium-labeled vitamin D [65]. Furth rmore, polar metabolites of vitamin D were found by Haussler, Myrtle, and Norman [66]. The Wisconsin group labeled these metabolites as peak 5 [64], the Norman group called it peak 4B [66], and Lawson, Wilson, and Kodicek d - scribed it as peak P [65]. Kodicek *et al.* claimed that the metabolite of vitamin D found in intestine was deficient in tritium at the 1 position [65]. However, Myrtle *et al.* reported that peak 4B did not lose its tritium [67]. Thus, the suggestion of a modification at the 1 position could not be confirmed. The DeLuca group, however, isolated the active metabolite from intestines of 1600 chickens given radiolabeled vitamin D, and, by means of mass spectrometric techniques and specific chemical reactions, the structure of the active form of vitamin D in the intestine was unequivocally demonstrated as $1,25(OH)_2D_3$ [68]. Of great importance was the finding of Fraser and Kodicek that the peak P metabolite could be produced by homogenates of chicken kidney and that anephric animals are unable to produce the p ak P metabolite [69]. They correctly concluded that the site of synthesis of the active form of vitamin D is the kidney. The Wisconsin group then chemically synthesized both $1\alpha,25(OH)_2D_3$ [70] and $1\beta,25(OH)_2D_3$ [71] and provided unequivocal proof that the active form is $1\alpha,25(OH)_2D_3$. Furthermore, this group was able to synthesize $1\alpha OHD_3$, an important analog that assumed great importance as a therapeutic agent throughout the world [72].

D. Proof That $1,25(OH)_2D_3$ Is the Active Form of Vitamin D

Proof that $1,25(OH)_2D_3$ and not $25OHD_3$ is the active form was provided by experiments in which anephric animals respond to $1,25(OH)_2D_3$ by increasing intestinal absorption of calcium and bone calcium mobilization, whereas animals receiving $25OHD_3$ at physiological doses did not [73–75]. Furthermore, the experiment of nature, namely, vitamin D-dependency rickets type I, an autosomal recessive disorder, provided final proof [76]. This disease could be treated by physiological doses of synthetic $1,25(OH)_2D_3$, whereas large amounts of vitamin D_3 or $25OHD_3$ were needed to heal the rickets. Although the exact defect in this disease is yet unknown, it is believed to be a defect in the 1α-hydroxylase enzyme that converts 25OHD to the final active form (see Chapter 47). $25OHD_3$ at pharmacological doses likely acts as an analog of the final vitamin D hormone, $1,25(OH)_2D_3$ (Fig. 2).

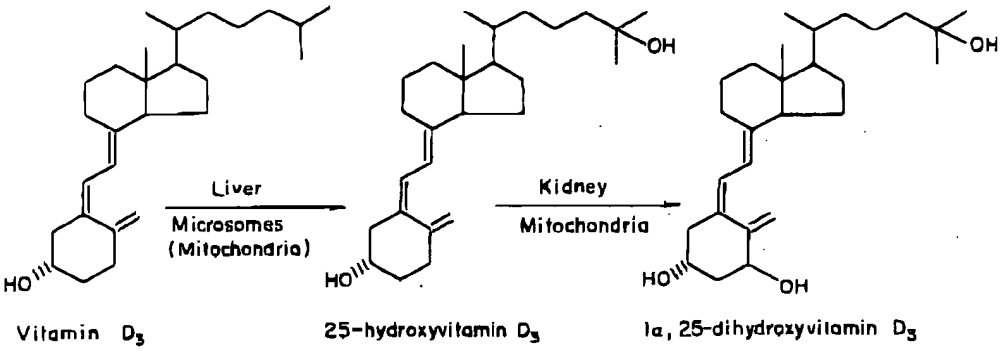


FIGURE 2 Activation of the vitamin D_3 molecule.

E. Discovery of the Vitamin D Endocrine System

Immediately after the identification of $1,25(OH)_2D_3$ as the active form of vitamin D came studies in which it could be shown that animals on a low calcium diet produce large quantities of $1,25(OH)_2D_3$, whereas those on a high calcium diet produce little or no $1,25(OH)_2D_3$ [77]. A reciprocal arrangement was found for the metabolite $24R,25(OH)_2D_3$. Thus, when calcium is needed, production of $1,25(OH)_2D_3$ is markedly stimulated and the 24-hydroxylation degradation reaction is suppressed. When adequate calcium is present, production of $1,25(OH)_2D_3$ is shut off and the 24-hydroxylation reaction is turned on. This discovery also satisfactorily provided evidence that $1,25(OH)_2D_3$ is the likely endogenous factor originally described by Nicolaysen *et al.* [34].

The next important step was the demonstration that it is parathyroid hormone that activates 1α-hydroxylation of $25OHD_3$ in the kidney [78]. Thus, parathyroidectomy eliminates the hypocalcemic stimulation of 1α-hydroxylation and suppression of 24-hydroxylation, whereas administration of parathyroid hormone restores that capability. Fraser and Kodicek also provided evidence that, in intact chickens, injection of parathyroid hormone stimulated the 1α-hydroxylation reaction [79]. Thus, the basic vitamin D endocrine system was largely discovered and reported in the early 1970s, being completed by 1974.

F. Other Metabolites of Vitamin D

During the course of identification of $1\alpha,25(OH)_2D_3$, $21,25(OH)_2D_3$ was reported as a metabolit , as was $25,26(OH)_2D_3$ [80,81]. However, the identification of $21,25(OH)_2D_3$ was in error and was corrected to $24,25(OH)_2D_3$, with the correct stereochemistry as $24R,25(OH)_2D_3$ [82]. Over the late 1970s and early 1980s, as many as 30 metabolites of vitamin D were identified [83]. These are covered in other chapters in this volume. Of great importance was the use of the fluoro derivatives of vitamin D to illustrate that the only activation pathway of vitamin D is 25-hydroxylation followed by 1-hydroxylation [84]. Thus, 24-difluoro-$25OHD_3$ supported all known functions of vitamin D for at least two generations of animals [85]. 24-Difluoro-$25OHD_3$ cannot be 24-hydroxylated. Furthermore, other fluoro derivatives such as 26,27-hexafluoro-$25OHD_3$ [86] and 23-difluoro-$25OHD_3$ [87] are all fully biologically active, illustrating that 26-hydroxylation, 24-hydroxylation, and 23-hydroxylation are not essential to the function of vitamin D.

G. Discovery of the Vitamin D Receptor

Zull and colleagues provided evidence that the function of vitamin D is blocked by transcription and protein inhibitors [88]. Thus, it became clear very early that a nuclear activity is required for vitamin D to carry out its functions. This work confirmed and extended the earlier work of Eisenstein and Passavoy [89]. With the discovery of the active forms of vitamin D came new attention to the idea that vitamin D may work through a nuclear mechanism. Thus, Haussler *et al.* reported vitamin D compounds to be associated with chromatin [66]. However, these experiments did not exclude the possibility that the vitamin D compounds might be bound to the nuclear membrane. The first clear demonstration of the existence of a vitamin D receptor was at the hands of Brumbaugh and Haussler [90]. Furthermore, the experiments of Kream *et al.* [91] provided strong and unequivocal evidence of the existence of a nuclear receptor for $1,25(OH)_2D_3$. Intense efforts toward purification of the receptor and its study appeared with the knowledge that it is an approximately 55,000

molecular weight receptor protein. In 1987, a partial cDNA sequence for the chicken vitamin D receptor was determined [92]. This was followed by isolation of the full coding sequence for the human [93] and rat [94,95] receptors.

From a historical point of view, one of the most important discoveries was vitamin D-dependency rickets type II [96], which is now known to be due to a defect in the receptor gene [97,98] (discussed in Chapter 48). This discovery essentially provided receptor knockout experiments in humans, allowing unequivocal proof of the essentially of the vitamin D receptor for the function of vitamin D. The nature of the receptor and how it functions are described in subsequent chapters along with current thinking on the molecular mechanism of action of $1,25(OH)_2D_3$.

Acknowledgments

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